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13. SUPPLEMENTARY NOTES

14. ABSTRACT: The main purpose of the proposed study was to evaluate the toxicity and efficacy of multiple targeting vectors for the treatment of prostate cancer in mouse models. It included Avastin, an anti-vascular endothelial growth factor (VEGF) antibody and PAI2, a protein that targets uroplasminogen activation (uPA) receptors in the PA system; both to be used in combination therapy together with labeling with alpha-emitting radionuclide, Bismuth-213. New methods for the labeling of Avastin were developed with labeling efficiency in excess of 90%. The resulting conjugate was tested for in vitro stability and was found to be stable with less than 20% leaching over a period of 5 half-lives of the isotope. Enhancement of plasminogen activation expression was achieved with VEGF with PC3 cell line showing the maximum enhancement, DU145 showing some enhancement whereas LN3-LNCaP showing no change in its other negative uPA expression. VEGF secretion was estimated for the three cell lines and based on that PC3 was chosen for the tumour model development. Subcutaneous and orthotopic tumour models were successfully developed in mice and are subject of the ongoing in vivo studies. Successful labeling and stability testing of the Avastin conjugate is world first and remains the most significant finding of the study to-date.

15. SUBJECT TERMS
Tumour model development, anti-VEGF monoclonal antibody Avastin, combination therapy, radiolabeling, radioimmunoconjugate, stability, uPA expression, PAI2, Bismuth-213

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INTRODUCTION

The Award involves the combination of the anti-vascular endothelial growth factor (VEGF) therapy with targeted alpha therapy (TAT). It combined the existing TAT skills of the PI with another targeting approach (anti-VEGF therapy) that has been used against various cancers. A brief introduction to the proposal is given below:

Solid tumours require their own vascular systems for progressive growth and the production of angiogenic growth factors by tumour cells is necessary to induce their functional tumour vasculature[1,2]. VEGF is one of the most important growth factors and possesses strong angiogenic activity in both in vitro and in vivo assays and also is responsible for the formation of vascular hyperpermeability, ascites and edema[3,4]. The presence of this angiogenic growth factor in a number of tumours has been demonstrated, and VEGF expression is up-regulated in hypoxic tumour areas[5-7] and is expressed at high levels in other cancers[8-11]. In prostate cancer, the degree of tumour vascularization correlates with the development of metastatic disease[12]. Though the critical factors that promote angiogenesis in prostate cancer are yet to be defined, Melnyk et.al[13] found that VEGF promotes tumour angiogenesis in prostate cancer. Utilizing a VEGF-neutralizing monoclonal antibody[14], they examined the effect of VEGF inhibition in an in vivo model of metastatic prostate cancer. They found that inhibition of VEGF suppresses both primary prostatic tumour growth and dissemination of micrometastases. In addition, they also found that VEGF appeared to be inhibiting further growth and metastatic progression of well established tumour grafts. For prostate cancer, however, the anti-VEGF therapy has not been too successful and it needed to be combined with other therapeutic approaches and TAT was considered as one of them. The PI proposed to use the FDA approved and humanized anti-VEGF monoclonal antibody, Avastin, for this purpose. The proposal comprises a comprehensive regime of in vitro and in vivo experiments to test the proposed hypothesis that stated "Vascular endothelial growth factor receptors (VEGFRs) are expressed by most prostate cancer patients that can be targeted by the commercially available anti-VEGF monoclonal antibody labelled with Bismuth-213 alpha emitter and the remainder can be eliminated by Bismuth-213 labeled plasminogen activator inhibitor type 2 (PAI2)". Targeting has been the main pillar of the study, but the approach is two-fold. Blocking the VEGFRs by anti-VEGF therapy (labelled and unlabeled) inhibits tumour proliferation and this phenomenon will be tested in combination with TAT.

BODY

The two-year study required a series of Tasks to be performed with an appropriate timeline for each Task. Below is a description of the work carried out to-date and is based on the approved Statement of Work (SOW) tasks and milestones as outlined in the proposal.

Task 1 Test and develop the methods for the in vitro experiments that includes testing of the prostate cancer cell lines (PC3, LN3-LNCaP and DU145) for VEGF secretion, detection of an enhancement of plasminogen activation (PA) expression by VEGF and labelling of Avastin with Bismuth-213. The cell lines that have maximum VEGF and VEGFR levels will be used for the development of prostate cancer animal models. The in vitro experiments will lead to selection of appropriate tumour models and labelling of Avastin that will be used for subsequent in vivo experiments. The approximate time required to complete these experiments is 3-6 months.

Progress / Accomplishments The Task was completed within the time frame proposed. Following experiments were carried out to complete the Task:

1 The three cell lines were grown as proposed in the protocol and spectrophotometric assay of the VEGF secretion by each of the cell line was carried out by following the Quantakine Elisa kit instructions. A number of experiments were carried out to optimize / standardize the procedure. Results from one of the experiments (after optimization / standardization of the procedure) are provided as an example.
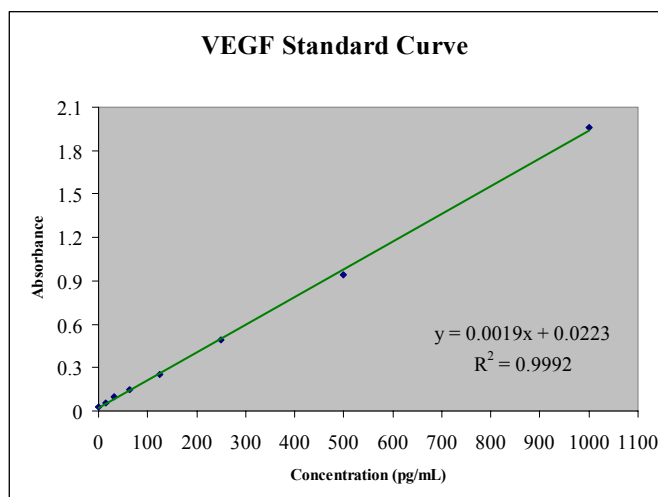

Figure 1 VEGF Standard Curve

Figure 1 represents the VEGF standard curve prepared from the known VEGF standard concentrations. The Table 1 provides the calculated concentrations of the unknown and blind known standard/s test samples.

Table 1   Data for standard curve and calculated values of the VEGF in unknown specimens

Standard			Description	DU145		LN3-LNCaP		PC3	
Absorbance	**Concentration (pg/mL)**			Abs.	Conc.	Abs.	Conc.	Abs.	Conc.
	Known	**Calculated**							
0.029	0	3.5	Undiluted sample (S)	1.063	547.7	0.234	-7.5	0.682	347.2
0.059	15.6	19.3							
0.097	31.2	39.3	Dilution 1 1:5	0.270	130.4	0.044	11.4	0.118	50.4
0.0147	62.5	65.6							
0.255	125	122.5							
0.49	250	246.2	Dilution 2 1:10	0.141	62.5	0.008	111.4	0.040	9.3
0.94	500	483.0							
1.957	1000	1018.3							

The cell line DU145 showed the highest VEGF secretion, but it has shown to be relatively difficult in longer term monitoring of the tumour growth that we encountered in some previous tumour model development studies. LN3-LNCaP cell line was ruled out because of the lack of VEGF secretion in these experiments. It was therefore, decided that PC3 cell line will be used for the development of animal models for this study.

3   The enhancement of uPA expression was tested by growing and incubating the three cell lines with VEGF. For this purpose $VEGF_{165}$ and $VEGF_{121}$ were used at varying concentrations. It was found that uPA expression was enhanced by VEGF and the enhancement was consistent for both types of VEGF. It is clear from Figure 2 that uPA expression enhancement was highest in case of the PC3 cell lines [PC3 cell line is ++ positive for uPA expression] but was independent of the concentration and type of VEGF used. The expression was increased from ++ to +++ and ++++ with VEGF treatment. For DU145 cell line, there was some enhancement in uPA expression that was independent of the type and concentration of the VEGF used. Figure 3 shows this enhancement phenomenon. The cell line is + to ++ (depending on the condition of the cells growing, passage number and the antigen expression), but when treated with either VEGF, it becomes ++ to +++ as observed in some slides (not shown).

The cell line LN3-LN-CaP is uPA negative. The enhancement of the uPA by VEGF was not observed in this cell line and it was true for each concentration of the VEGF regardless of the type [although some positive cells were observed in some slides, but later these were classified as false positives]. The Figure 4 shows the lack of uPA expression in LN3-LNCaP cell line, when subjected to treatment with various concentrations of $VEGF_{165}$ and $VEGF_{121}$.
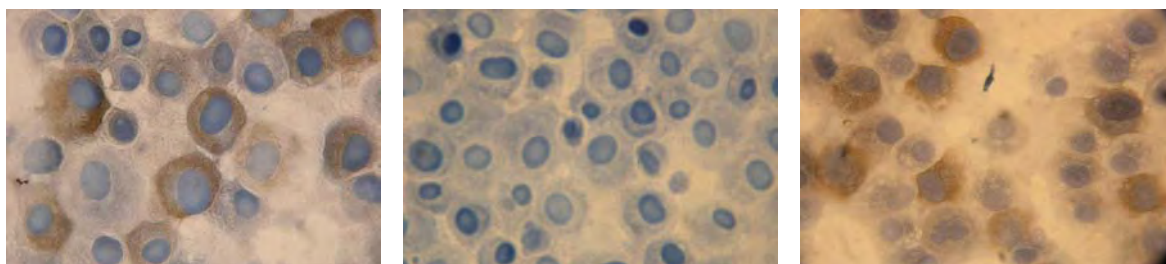
Figure 2   Enhancement of uPA expression by VEGF for PC3 cells. Positive control (Left), Negative control (middle), PC3 cells treated with $VEGF_{165}$ (right).
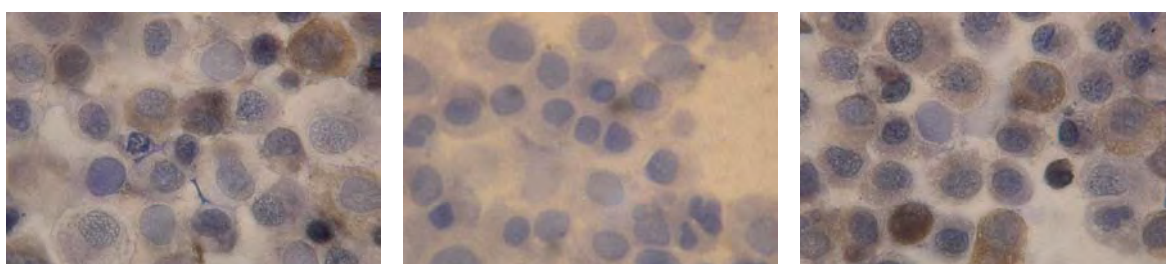

Figure 3   Enhancement of uPA expression by VEGF for DU145 cells. Positive control (Left), Negative control (middle), DU145 cells treated with $VEGF_{121}$ (right).

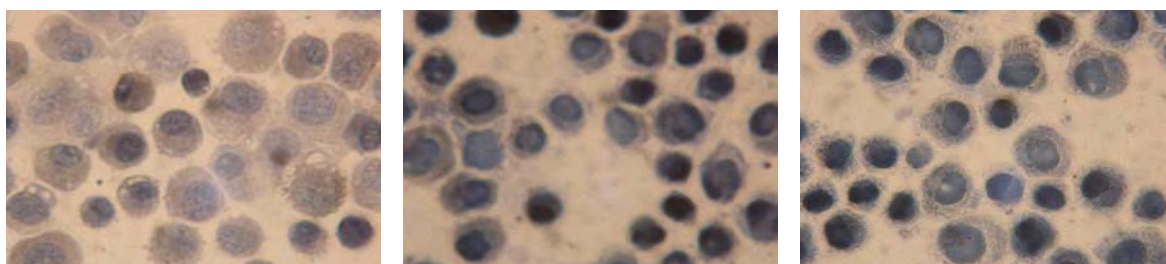**Figure 4**   Lack of uPA expression by VEGF for LN3-LNCaP cells. Positive control (Left), Negative control (middle), DU145 cells treated with $VEGF_{165}$ (right). No uPA positive cells can be seen in any of the pictures shown.

4   The development of a method for the labeling of Avastin with Bismuth-213 was achieved as a world first in this study. It required numerous experiments to optimize the pH, time, reaction conditions, buffers and buffer exchange procedures that eventually lead to the successful labeling of Avastin. The following Figure shows the results of a series of labeling experiments that were carried out after the standardization of the procedure.
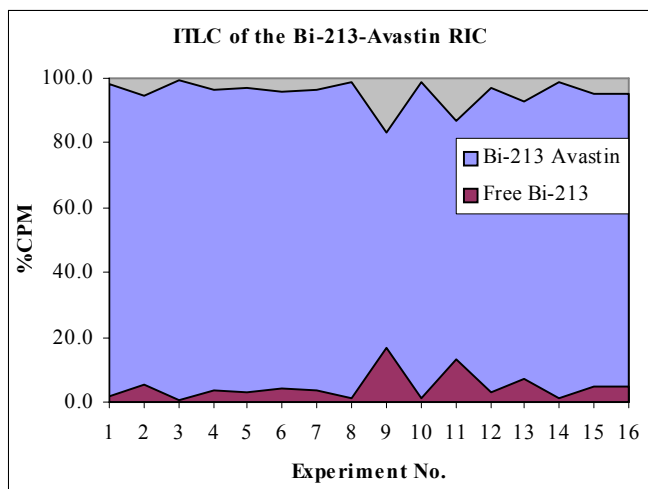
Figure 5 shows that the labeling efficiency of [213]Bi-Avastin RIC is >95%. The data presented in this Figure is based on a specific activity range of 185 – 800 MBq/mg but the optimum specific activity is yet to be determined, as it remains a major issue with respect to reduction in toxicity and enhancement of specific targeting. Experiments are being carried out to address this issue of specific activity and the initial experiments in this regard have been successful.

Figure 5. Standardization and testing of the procedure for the labeling of Avastin with the alpha emitting radionuclide [213]Bi.

5 Stability of [213]Bi-Avastin radioimmunoconjugate (RIC) The stability of the radioimmunoconjugate was tested by subjecting the conjugate to a DTPA challenge (using large excess of the DTPA) and serum stability test.
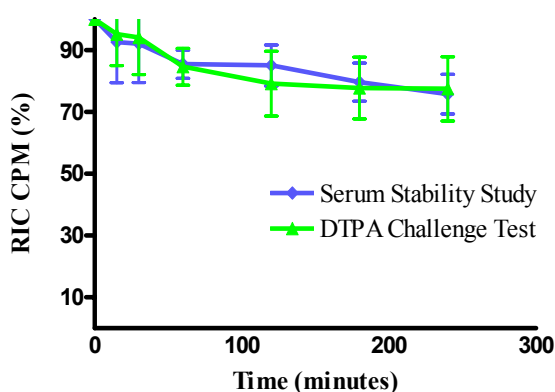

Figure 6 shows the results of the stability tests for [213]Bi-Avastin RIC. It is clear that more than 80% of the activity remains attached with the radioisotope up to 4 hours or 5 half-lives of [213]Bi. The data presented are based on a specific activity range of 185 – 800 MBq/mg but the optimum specific activity is yet to be determined as mentioned above.

Figure 6. DTPA challenge and serum stability tests of the Bi-Avastin RIC.

Experimental Objectives achieved The following experimental objectives as proposed in the original protocol were achieved by completing this Task 1:

1. In vitro measurements of the VEGF concentration in the prostate cancer cells that will be used for the in vitro and in vivo experiments of the proposed study and to develop an immunohistological procedure for the detection of VEGFRs in these cell lines by using Avastin.

2. To determine whether VEGF enhances the plasminogen activation (PA) expression in 3 cell lines.

3. To standardize the labeling procedure for Avastin with [213]Bi.

[*Experimental Objectives 1-3 achieved*].

Task 2 *Development of the animal models* The animal models will be chosen based on the in vitro experiments for the detection of VEGF and VEGFR. All three cell lines have been reported to secrete VEGF and VEGFR and if similar results are found, I plan to use all three models for the efficacy and toxicity of the combination therapy. Time line for development of animal models is 4-6 months.

Progress / Accomplishments The Task was completed within the time frame proposed as described below:

1. Using the PC3 cell line, the animal models were developed for subcutaneous and orthotopic xenografts.

2. Appropriate training for the orthotopic model was provided by Professor Pam Russell (one of the Mentors) prior to the actual cell inoculations. This included anaesthesia and recovery protocols, operating and locating the prostate, administration of appropriate volumes of saline in prostate and possible complications and common errors that are made during such procedures. Training for correct stitching of the two layers was also provided.

The Figure 7 shows the pictures of mice with subcutaneous tumours and the tumour from a mouse that was injected intra-prostatic PC3 cells.

Experimental Objectives achieved The following experimental objectives as proposed in the original protocol were achieved by completing this Task 2

1. To develop appropriate tumour xenograft models required for the study based on VEGF secretion and VEGFR expression by the cell lines tested in vitro.
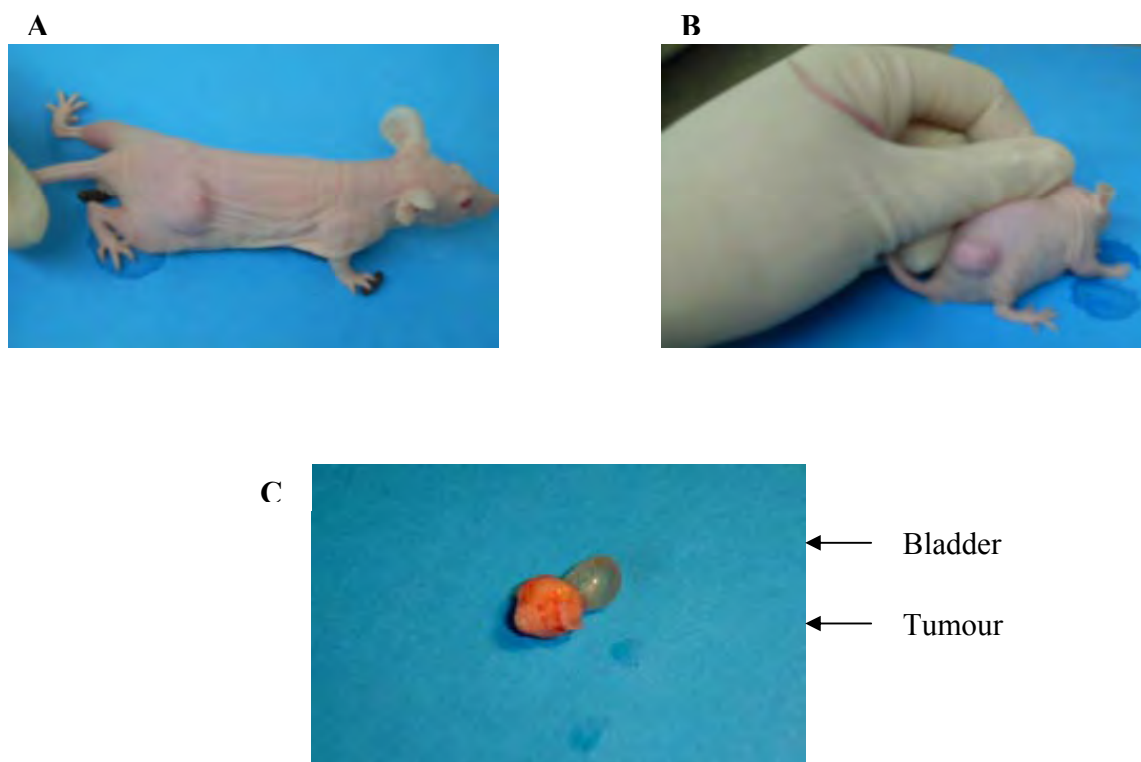
[*Experimental Objective 4 achieved*].

Figure 7.　　　Development of animal model: Subcutaneous (A and B) and Orthotopic (C). The mice were inoculated with PC3 cell line.

Task 3　　The efficacy and toxicity of Avastin has been reported in the literature. However, we will perform this experiment to compare it with Bismuth-213 labeled Avastin. This task does not involve the combination therapy and only one tumour model will be used. Timeline for the completion of the task and achievement of the milestone is 6-12 months.

Progress / Accomplishments　　　The Task was started within the time frame proposed in the protocol, but the final results are still being worked out as longer monitoring was required for some groups. A brief description of the work carried out to accomplish Task 3, is given below:

Three different doses of cold Avastin were administered in mice. The doses included a range from low (1 mg/kg) to high (5 mg/kg) with a medium range of 3 mg/kg. The tumour size of the mice at the start of Avastin administration ranged between 2-4 mm in any one direction [the range is provided for information only – the minimum tumour volume in each mouse was ensured to be at least 2 mm at the start of the Avastin therapy]. All mice were initially administered with the specified doses of cold Avastin at twice weekly for three weeks from the day of the first administration. As mentioned

earlier, while the final results are still not available for this Task, an overview of the data suggests that cold Avastin does have an effect but probably independent of the dose. It also suggests that, while the higher (5mg/kg) dose was probably not more efficacious than the medium dose, it certainly induced more toxic events in the mice [although the histopathology results are not yet available], as the mice looked more sick and uncomfortable at this dose compared to lower doses.

Tasks 4 and 5

Task 4 The efficacy and toxicity of the combination therapy, comprising cold Avastin and Bismuth-213 labeled PAI2, will be tested in one tumour model as this experiment does not include Bismuth-213 labeled Avastin. Timeline for the completion of this task is 12-18 months.

Task 5 This includes optimization of the time interval between the Bismuth-213 labeled Avastin and Bismuth-213 labeled PAI2 using one tumour model as for Tasks 3 and 4. Timeline for the completion of the task and achievement of the milestone is 9-15 months.

Progress / Accomplishments Tasks 4 and 5 are combined together as both were carried out in one experiment. The experiment is still ongoing and the final results / data are not yet available. A brief description of the experimental design, progress and accomplishments is given below:

1. The experimental design as proposed in the original protocol (Experiments 5-7) involved the dose range between 1.5 and 5 mCi/kg. Based on the more recent ^{213}Bi labeled antibodies / proteins long-term toxicity studies, it was decided to use one low (3 mCi/kg) and one high (9 mCi/kg) dose of either of the Avastin or PAI2 labeled with ^{213}Bi.

2. The total dose was reduced for each of the radiolabeled products to half to ensure that the total radiation dose delivered does not exceed the upper limit of 9 mCi/kg.

3. Time interval optimization experiment was modified to include the optimization of time interval between radiolabeled and unlabeled Avastin. Time interval optimization for the ^{213}Bi-labeled Avastin and PAI2 was not required, as both were administered at the same time (at reduced doses as described in 'b' above).

Results The data are not yet available, but it is expected that the results from this set of experiments will be presented at the IMPaCT meeting in September 2007.

Experimental Objectives achieved The following experimental objectives as proposed in the original protocol were achieved by completing this Task 2

1.   To determine the efficacy and toxicity of cold Avastin and ^{213}Bi-Avastin in one tumour model.

2.   To determine the efficacy and toxicity of the combination therapy of the optimized dose of cold Avastin (from Experiment 5) and varying doses of ^{213}Bi-PAI2 in one tumour model.

3.   To determine the optimized time interval between ^{213}Bi-Avastin and ^{213}Bi-PAI2 administration for combination therapy using one tumour model.

[***Experimental Objectives 5-7 are being achieved***].

KEY RESEARCH ACCOMPLISHMENTS

The key research accomplishments to-date are given below:

- Successful standardization of a method for the quantitative estimation of Vascular Endothelial Growth Factor (VEGF) secreted by three prostate cancer cell lines.

- Enhancement of uroplasminogen activation (uPA) expression has successfully demonstrated.

- Tumour models (subcutaneous and orthotopic) have been successfully established.

- The biggest accomplishment to-date has been the successful labeling of Avastin with the alpha emitting radionuclide Bi-213 and testing the stability of the so-produced radioimmunoconjugate in vitro [a paper is being written on these results and is expected to be submitted in a few weeks time].

- The time interval between cold and radiolabeled Avastin has been successfully established.

- The combination therapy dose and interval optimization experiments are currently ongoing.

- The work is going on as per plan and is expected to be complete within the stipulated time period of two years from the start date.

REPORTABLE OUTCOMES

The major reportable outcome since the start of the grant has been the successful labeling and testing of the anti-VEGF monoclonal antibody Avastin. The specific activity work is being carried out alongside the in vivo stability studies. The labeling data however, in itself is expected to form a full-fledged refereed research paper that is being written and is expected to be ready in few weeks time.

Based on the results of the current work carried out to-date, a fresh application has been lodged with the United States Department of Defense (USDoD), Prostate Cancer Research Program (PCRP). The new application primarily targets multiple vectors for prostate cancer.

The early results of the ongoing research work clearly demonstrate the superiority of the multiple targeting achieved in case of cold and hot Avastin, cold Avastin and ^{213}Bi-PAI2 and the hot Avastin and ^{213}Bi-PAI2 combination therapies compared to Avastin only (cold or hot) and ^{213}Bi-PAI2 only mono-therapies.

The new application also involves the targeting of the developed vasculature through vascular disrupting agent (VDA) BNC-105. This ongoing work together with the proposed work in new application is expected to demonstrate the superiority of the combination therapy with multiple vectors proposed and will generate more than adequate data to start combination therapy clinical trials for prostate cancer.

CONCLUSION

Avastin has been and thus can be successfully labeled with radionuclides that can be used to enhance the efficacy and targeting of cold Avastin. Combination therapy certainly seems to be a better option compared to Avastin based mono-therapy [as evidenced from the ongoing animal studies].

It is however, recommended that following studies should be carried out to ensure validity and completeness of the data with respect to the initiation of early Phase clinical trials for prostate cancer using the proposed therapy:

Optimization of specific activity (increased specific activity will require less amounts of the antibody and thus the target blocking effect of the antibody will be reduced resulting in an increased targeting by the antibody),

The accuracy of pharmacokinetics studies is extremely important and we have time and again observed that such studies with the short half-lived ^{213}Bi provide only a general trend rather than accurate pharmacokinetics. It is therefore, suggested that ^{205}Bi / ^{206}Bi radionuclide be used for these studies (preliminary experiments have already been carried out and results are being analysed),

Toxicity studies should essentially be carried out in a second species and it is proposed by the medical oncologists in our Institution that rabbits be used for this purpose. A data from mice and rabbits for acute, short-term and long-term toxicity will provide sound basis for a human clinical trial.

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